

**Fig. 4.** Analysis of tissue sections from experimental shigellosis in rabbits (A to D) and spontaneous human appendicitis (E to H). (A) Immunofluorescence staining of histones reveals nuclear and extracellular localization that largely overlaps with staining for DNA (C). (B) Staining with an antibody against *Shigella*-specific LPS. (D) The overlay indicates that numerous *Shigellae* are closely associated to

fibrous material staining for histones and DNA. (E) Staining for neutrophil elastase in an area of neutrophil exudate in human spontaneous appendicitis reveals fibrous extracellular material that also stains for histone (F) and DNA (G). (H) Overlay of the images. The images are projections of confocal z stacks generated from sections of 5 to 6  $\mu\text{m}$  thickness. Bar, 50  $\mu\text{m}$ .

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#### Supporting Online Material

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## Emerging Vectors in the *Culex pipiens* Complex

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In the Old World, some mosquitoes in the *Culex pipiens* complex are excellent enzootic vectors of West Nile virus, circulating the virus among birds, whereas others bite mainly humans and other mammals. Here we show that, in northern Europe, such forms differing in behavior and physiology have unique microsatellite fingerprints with no evidence of gene flow between them, as would be expected from distinct species. In the United States, however, hybrids between these forms are ubiquitous. Such hybrids between human-biters and bird-biters may be the bridge vectors contributing to the unprecedented severity and range of the West Nile virus epidemic in North America.

Species in the *Culex pipiens* complex are considered to be the primary vectors of West Nile virus (WNV) in North America because they are often the most common mosquitoes in urban areas (1), because disease outbreaks occur during their peak abundance period (2), because they are competent laboratory vectors of WNV (3), and because field populations in the United States have repeatedly been found infected with the virus (4, 5). In addition, they can transmit the virus transovarially (6), so

overwintering mosquitoes can serve as a source of WNV to initiate an infection cycle in the spring (7). Blood-meal analysis has revealed that *Cx. pipiens* in the United States bite both humans (anthropophagy) and birds, suggesting they may serve as bridge vectors of the disease from birds to humans (2). Human WNV epidemics require bridge vectors, because humans and other mammals do not usually generate high enough viremia to infect biting mosquitoes (8). Although *Cx. pipiens* has been

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implicated in recent deadly urban outbreaks of WNV in Europe, these were nonrecurrent and localized (8). The persistence and spread of WNV in the United States have therefore been very surprising, and an in-depth examination of different populations of *Cx. pipiens* worldwide was warranted.

The *Cx. pipiens* complex has been considered "one of the major outstanding problems in mosquito taxonomy" (9), because a markedly divergent array of physiological and behavioral traits occurs without distinctive morphological differentiation (1, 10). Two species are currently formally recognized in the complex: *Cx. pipiens* Linnaeus 1758 and *Cx. quinquefasciatus* Say 1823, the northern and southern house mos-

quitoes that are ubiquitous in temperate and tropical regions, respectively (11). Their geographical distributions overlap closely with that of humans, who introduced them into many areas (1).

Most specimens of the temperate species, *Cx. pipiens*, require a blood meal for egg development (anautoecy) and overwhelmingly bite birds (12). In northern European cities, however, some underground populations, such as those in the London underground railway tunnels (12), can lay a first batch of eggs without a blood meal (autoecy), although they will bite humans readily and ravenously given the opportunity (13). These same underground populations can also breed in confined spaces (stenogamy), whereas aboveground *Cx. pipiens* mate while swarming in a large breeding area. Underground populations remain active throughout the year, whereas northern aboveground populations of *Cx. pipiens* go into torpor during the winter (diapause) (1).

One interpretation of the existence of these two distinct strategies in *Cx. pipiens* is that rapid shifts in physiological and behavioral traits are the consequence of local adaptation to underground environments associated with human activity (14). Indeed, a study comparing underground and aboveground populations in London using allozymes concluded that the underground autogenous populations were de-

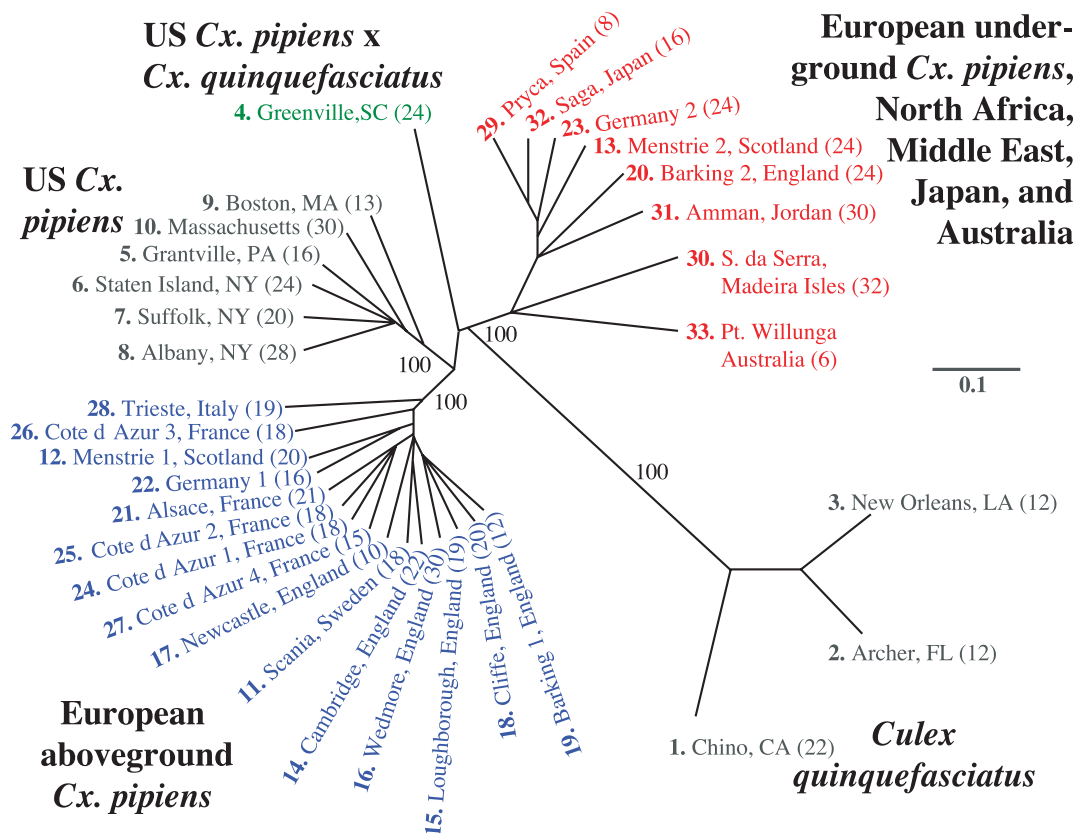
rived from those aboveground. However, the authors found such high differentiation and mating barriers between the two physiological forms that they were forced to hypothesize that a single colonization event was responsible for all the underground populations in London (12).

An alternative interpretation for the existence of two behavioral and physiological forms is that they correspond to two separate species (i.e., genetically distinct entities). A predominantly northern species, *Cx. pipiens*, would be a bird-dependent autogenous mosquito that diapauses and needs open spaces to mate. Its southern counterpart, *Cx. molestus* [presently a synonym of *Cx. pipiens* (9, 13)], would not diapause and would be stenogamous. In this scenario, the northern underground populations are derived from autogenous *Cx. molestus* of southern Europe and northern Africa, which colonized northern countries as warm and nutrient-rich underground facilities were created (14). In southern populations, both autogenous and anautogenous individuals coexist (9, 13), but autogeny is commonly associated with a preference for mammals and in particular anthropophagy (1, 14). Indeed, anthropophagy was the character that originally led to the description of *Cx. molestus* Forskal 1775 from autogenous Egyptian specimens, although this species was later synonymized under *Cx. pipiens*

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**Fig. 1.** Unrooted distance tree, based on proportion of shared alleles, depicting the relationships between the 33 populations used in this study. Numbers in parentheses indicate the number of individuals sampled, and numbers on branches indicate bootstrap values. The numbers before each name correspond to the numbers in Fig. 2.





because of the lack of diagnostic morphological characters (13).

Critically, these two hypotheses generate different predictions about genetic similarity among autogenous and anautogenous populations. If northern European underground autogenous populations evolved from aboveground *Cx. pipiens*, then they should be genetically more closely related to nearby aboveground anautogenous populations than to autogenous populations in other geographic areas. If, however, underground autogenous populations belong to a different species, then all autogenous populations should constitute a monophyletic group.

We tested these hypotheses using highly polymorphic microsatellite markers (15) developed for species in the *Cx. pipiens* complex (16, 17). We used an array of eight microsatellite loci that amplify consistently across all populations tested (CQ11, CQ26, CxqGT4, CxqGT6b, CxpGT4, CxpGT9, CxpGT12, and CxpGT46), to fingerprint autogenous (underground) and anautogenous (aboveground) populations of *Cx. pipiens* from Britain and Germany, as well as other aboveground populations from Britain, Sweden, northern and southern France, Italy, northern Africa, the Middle East, Japan, Australia, and the United States (tables S1 to S2). Only autogenous forms are known to have been introduced to Japan and Australia (18, 19). We also analyzed *Cx. quinquefasciatus* from southern localities in the United States as an outgroup (Fig. 1). The similarity between autogenous mosquitoes from different geographic areas and their differentiation from sympatric or geographically close populations of aboveground mosquitoes was apparent from the standard distance measures (Fig. 1), as well as from the allelic frequency distributions (tables S1 to S2). Northern European aboveground and underground (autogenous) populations formed two distinct clusters. Underground populations clustered with North African and Middle Eastern populations as well as with the autogenous populations from Japan and

Australia. Significantly, the northern European underground populations have a lower average number of alleles per locus than the aboveground populations (alleles  $\pm$  SE =  $2.06 \pm 0.20$  and  $7.64 \pm 0.43$ , respectively,  $P < 0.01$ , averaging across both loci and populations). Their most common alleles are also those that are common in African and Middle Eastern populations and are often nonexistent in northern European aboveground populations (supporting online material). Our results therefore reject the hypothesis that northern European autogenous mosquitoes result from local episodes of adaptation to underground environments. Instead, they indicate that northern European autogenous underground mosquitoes are a separate genetic entity, most likely derived from a southern species.

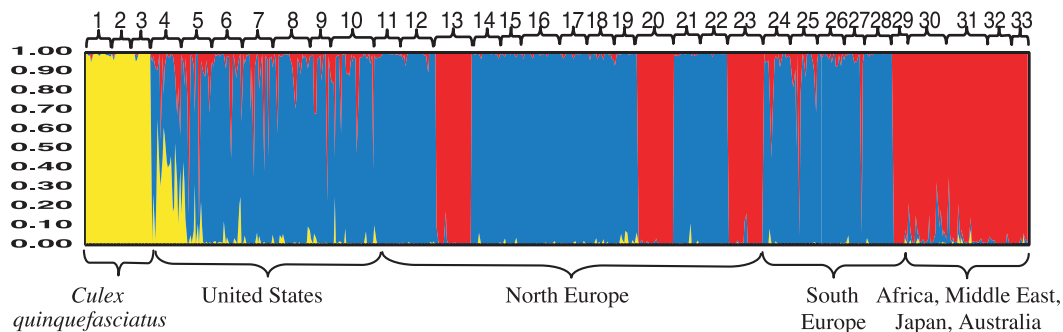
In the genetic distance analysis, U.S. *Cx. pipiens* populations clustered separately (Fig. 1). To examine in more detail the relationship between the North American populations and all remaining populations, we performed a multilocus genotype analysis that did not use prior information about the origins of individuals. Instead, it combined all the available individual multilocus genotypes and calculated the likelihood of a predetermined number of clusters ( $K$ ), assuming that some admixture (hybridization) might take place (i.e., we did not assume genetic isolation). We obtained the most likely  $K$  value from the examination of the range of likelihoods and their distribution (20) and plotted the probability of ancestry from each cluster for each individual (Fig. 2).

Using this technique, we identified three ( $K = 3$ ) most likely distinct genetic clusters in the 641 specimens included in the analysis (Fig. 2, the same specimens as in Fig. 1). These correspond to cluster A, which includes the overwhelming majority of European aboveground populations; cluster B, which includes autogenous specimens; and cluster C, *Cx. quinquefasciatus*. This analysis shows that all U.S. populations of *Cx. pipiens* include hybrids, which we de-

fine as specimens with a probability of ancestry equal to or higher than 0.06 from more than one cluster (more than one color in Fig. 2). On average, more than 40% of the specimens in each U.S. local population had hybrid ancestry from cluster A and cluster B. No significant number of hybrids was found in northern European populations. A small number of hybrids were found in two of the six southern European populations (10.2% per population on average) (Fig. 2). Examined separately, northern and southern European populations did not differ in allelic diversity ( $7.64 \pm 0.43$  and  $7.9 \pm 0.46$ , respectively), whereas U.S. *Cx. pipiens* have a significantly lower average number of alleles per locus and per population ( $5.90 \pm 0.30$ ,  $P < 0.01$ ). The U.S. allelic depletion is consistent with the idea that New World populations of *Cx. pipiens* were introduced from the Old World (1). We included in the analyses a population from South Carolina, a region where hybridization between *Cx. pipiens* and *Cx. quinquefasciatus* is prevalent (Fig. 2), to show that the uniqueness of U.S. *Cx. pipiens* populations is not due to the documented hybridization with *Cx. quinquefasciatus* (21–23). The South Carolina population clusters away from the core U.S. *Cx. pipiens* (Fig. 1).

The separation between the two behavioral and physiological forms of *Cx. pipiens*, which for the sake of brevity we will herein refer to as *Cx. pipiens* and *Cx. molestus*, is probably quite recent. The microsatellite signatures of *Cx. pipiens* and *Cx. molestus*, though distinct, are similar when compared to *Cx. quinquefasciatus*, for example (tables S1 to S8). *Cx. molestus* might be a commensal form that arose in association with the establishment of human settlements after the advent of agriculture (14) or by isolation during the Pleistocene glaciations. Both scenarios would place the separation at  $\sim 10,000$  years ago (24, 25), which could explain why more slowly evolving allozyme and morphological traits do not always distinguish the two forms (1, 12, 26, 27).

**Fig. 2.** Results of a Bayesian cluster analysis. Each of the 641 individuals included in the analysis is represented by a thin vertical line, partitioned into three colored segments that represent the individual's probability of belonging to one of the three genetic clusters (yellow, *Cx. quinquefasciatus*; blue, *Cx. pipiens*; red, *Cx. molestus*). For this representation, specimens were grouped by location (bracketed), and the location numbers are the same as in Fig. 1. The two long red lines within southern France populations 25 and 27 are specimens that have a probability of ancestry from *Cx. molestus* of more than 0.95 and are not considered hybrid.



What led to the pervasiveness of hybrids between *Cx. pipiens* and *Cx. molestus* in North America, but not in Europe and Africa, still remains to be determined. In southernmost Europe, we identified two populations with a few hybrid individuals, as well as populations with pure *Cx. pipiens* signatures and populations with a mix of pure *Cx. pipiens* and pure *Cx. molestus* signatures (Fig. 2). Indeed, previous allozyme-based studies indicated the existence of populations in Italy with a mix of the two forms (26) but a very low rate of hybridization (1%), probably because of their different mating behaviors (26). The rarity of southern European hybrids and our failure to find hybrids in northern Europe may be due to their low fitness and inability to diapause. Importantly, the introduction to the United States of separate populations of *Cx. pipiens* and *Cx. molestus* that later hybridized, or of hybrids from southern Europe, has led to abundant and ubiquitous hybrid forms that survive the rigors of northern winters.

It is now clear that models derived from the U.S. epidemic of WNV (28) may not be applicable to Eurasia, and vice versa (29). A major factor in all recent outbreaks (Romania 1996, Russia 1999, and United States 1999) is the involvement of mosquitoes in the *Cx. pipiens* complex as the primary vectors (8, 30). Unlike European *Cx. pipiens*, U.S. *Cx. pipiens* appears to bite readily both avian hosts and humans (2, 31). Here we have shown that, across the northeastern United States, a large proportion of individuals are hybrids of human-biter and bird-biter forms. In combination with susceptible migrating birds and highly concentrated human populations in U.S. cities and suburbs, the prevalence of such bridge vectors that readily transmit the virus among and between avian hosts and humans could have created the current epidemic conditions.

The present study suggests that changes in vectorial capacity and the creation of new efficient vectors may occur with new introductions. In particular, the arrival of hybrid American forms in northern Europe has the potential to radically change the dynamics of WNV in Europe.

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## Supporting Online Material

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Materials and Methods

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# Superfamilies of Evolved and Designed Networks

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Complex biological, technological, and sociological networks can be of very different sizes and connectivities, making it difficult to compare their structures. Here we present an approach to systematically study similarity in the local structure of networks, based on the significance profile (SP) of small subgraphs in the network compared to randomized networks. We find several superfamilies of previously unrelated networks with very similar SPs. One superfamily, including transcription networks of microorganisms, represents "rate-limited" information-processing networks strongly constrained by the response time of their components. A distinct superfamily includes protein signaling, developmental genetic networks, and neuronal wiring. Additional superfamilies include power grids, protein-structure networks and geometric networks, World Wide Web links and social networks, and word-adjacency networks from different languages.

Many networks in nature share global properties (1, 2). Their degree sequences (the number of edges per node) often follow a long-tailed distribution, in which some nodes are much more connected than the average

(3). In addition, natural networks often show the small-world property of short paths between nodes and highly clustered connections (1, 2, 4). Despite these global similarities, networks from different fields can have very different local structure (5). It was recently found that networks display certain patterns, termed "network motifs," at much higher frequency than expected in randomized networks (6, 7). In biological networks, these motifs were suggested to be recurring circuit elements that carry out key information-processing tasks (6, 8–10).

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